



Comparison of monocyte-derived dendritic cells from colorectal cancer patients, non-small-cell-lung-cancer patients and healthy donors

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ABSTRACT

Dendritic cells (DCs) are bone marrow-derived professional antigen presenting cells. Due to their role as potent inducers of immune responses, these cells are widely used as adjuvant in experimental clinical settings for cancer immune therapy. We have developed a DC-based vaccine using autologous blood monocytes loaded with allogeneic tumor cell lysate rich in cancer/testis antigens. This vaccine has at present been tested for activity in three phase II clinical trials including two cohorts of patients with advanced colorectal cancer (CRC) and one cohort of patients with advanced non-small-cell-lung-cancer (NSCLC). In the present paper we retrospectively compare the maturation profile based on surface marker expression on DCs generated from the three patient cohorts and between cancer patient cohorts and a cohort of healthy donors.

Vaccines were generated under cGMP conditions and phenotypic profiles of DC were analyzed by flow cytometry and the obtained data were used as a basis to set guideline values for our quality control of GMP produced DC vaccines. Each vaccine batch was analyzed for the expression of the surface maturation and differentiation molecules CD14, CD1a, CD83, CD86, MHC class II and CCR7, and the optimal expression pattern is considered as CD14^{low}, CD1a, CD83^{high}, CD86^{high}, MHC class II^{high} and CCR7^{high}.

In accordance with data from other studies including other types of cancer patients, especially breast cancer patients, we found that the maturation status of the DC batches depends on cancer type and correlates with clinical status of cancer patients included.

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1. Introduction

The immune system conducts surveillance and eradicates infections and controls early malignant growth. However, in clinical cases the cancer evades the immune system resulting in progression of the malignancy most likely leading to death of the patient. Defective functions of the immune system are thought to be an important mechanism by which tumors escape from the immune surveillance [1–3].

Professional antigen presenting cells (APCs) play a pivotal role in induction of antigen (Ag) specific immune responses including anti-tumor T cell immunity. Therefore dysfunctional APC could be partly responsible for some of the observed defective immune responses in tumor-bearing patients. The group of APC includes dendritic cells (DCs), macrophages and B cells, where DC is the most potent APC and the only APC that are capable of inducing primary immune responses [4,5]. Immature DC are found throughout

the body where they capture antigen for processing and presentation. Upon encounter with appropriate stimulation DC differentiate into mature DC. This transition to mature antigen presenting DC is defined by alteration of membrane bound protein expression: up-regulation of MHC classes I and II (antigen presenting molecules), CD80 and CD86 (co-stimulatory molecules) and CCR7 (chemokine receptor, a homing receptor for lymph nodes). By expression of CD86 and CD80 mature DC can activate naïve T cells leading to differentiation into cytotoxic T cells (CTL) capable of killing in an antigen specific manner [4–7].

It has been shown by several groups that T cells from cancer patients can be activated to the same level as healthy donor T cells if the DC used for stimulation are fully functional [8,9]. This indicates that the defective anti-tumor immune response observed in cancer patients might be due to defective DC. In agreement with this, defective DC isolated from cancer patients have been described. The major findings from these studies were that DC isolated from peripheral blood and lymph nodes from cancer patients displayed decreased expression of MHC class II and co-stimulatory molecules, and T cell stimulatory capacity was impaired [8,10–12].

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The existence of functionally defective DC in cancer patients strongly emphasizes the rationale of developing DC-based immune therapy to restore proper presentation of tumor associated antigens and T cell activation. Currently DC-based vaccines are being tested in various forms of cancers [6,13]. The most frequently used source of DC in DC-based vaccines is peripheral blood CD34⁺ progenitor cells or monocytes [14,15]. The “golden standard” for generating DC from blood monocytes is to culture monocytes with granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin 4 (IL-4) for 5–7 days, and subsequently another 1–2 days with maturation stimuli to generate a population of activated mature DC [6,16].

DanDrit Biotech A/S (DDB) has developed a vaccine (MelCancerVac[®], MCV) based on autologous monocyte-derived DCs (Mo-DC) loaded with cell lysate made from a selected melanoma cell line. This cell line has high expression of cancer/testis antigens that are over-expressed in many cancer forms (Weinert B, Manuscript submitted) [17,18]. DDB has conducted three phase II trials testing MCV, two with colorectal cancer (CRC) patients and one in non-small-cell-lung-cancer (NSCLC) patients.

In the present retrospective study we compare the maturation status of monocyte-derived DC generated from these three different cancer patient cohorts. To our knowledge this is the first time phenotypic status of autologous Mo-DC have been compared between CRC patients and NSCLC patients, and between such patient cohorts and healthy donors.

2. Material and methods

2.1. MelCancerVac[®]

2.1.1. First generation protocol

DC was generated accordingly to a modified protocol originally described by Romani et al. [15] and the modified protocol was described previously by Burgdorf et al. [19]. Briefly, PBMC from 200 ml of peripheral blood were isolated and after repeated washes the cells were resuspended according to the number of monocytes in appropriate amount of RPMI 1640 medium (Cambrex bio science) containing 1% of autologous plasma and plated for 1 h. Non-adherent cells were removed and the adherent fraction was cultured for 7 days. GM-CSF (100 ng/ml) and IL-4 (50 ng/ml) (Cell-Genix, Germany) were added on day 1, 3 and 5. On day 5 melanoma cell lysate (MCL) was added. On day 6 TNF α was added to induce maturation. On day 7 the cell culture was harvested and the number of DCs generated was determined.

3–5 $\times 10^6$ DCs were resuspended in 1 ml of physiological saline supplemented with 1% autologous plasma for injection. Remaining DCs were cryopreserved. Vaccines were manufactured according to cGMP requirements carefully regulated by SOPs.

2.1.2. Second generation protocol

As original protocol with some alterations: Maturation stimulus was expanded to consist of IL-1 β , IL-6, TNF α and PGE₂. In addition, the media used was AIM-V (Invitrogen Paisley, UK) for cell culture. One inclusion criterion was to generate at least three vaccines from 200 ml of peripheral blood. If this was not met the patients were leukapheresed, and the vaccine produced from the leukapheresis product.

2.2. FACS analysis

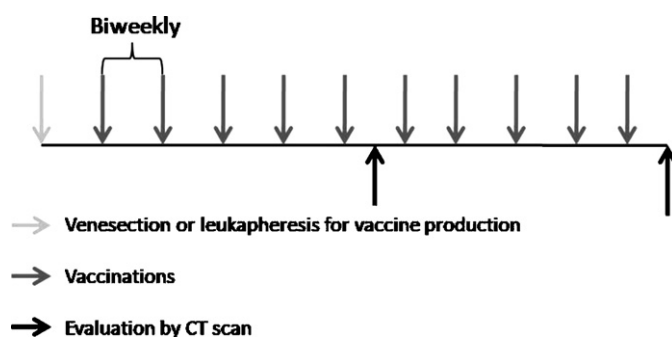
As previously described in [19]. Briefly, DC were harvested and stained with antibodies specific for the surface molecules: CD1a, CD14, CD83, CD86, HLA-DR, -P, -Q (all from Pharmingen Becton-Dickinson, Denmark) and CCR7 (R&D systems Europe, UK). Appropriate isotype controls were used. Samples were analyzed

using FACS Calibur Flow Cytometer (Becton-Dickinson) and CELLQuest software (Becton-Dickinson).

2.3. Clinical results

2.3.1. CRC Denmark

Twenty patients with progressing CRC stage IV showing a WHO performance status ≤ 2 were enrolled. However, only 17 patients received vaccine treatment. Data from this study were published recently [19]. In short, 10 vaccines were administered biweekly and before treatment, after 5 vaccines and after 10 vaccines the patients were evaluated by CT scan. Four stable diseases (SDs) were observed after the 5th vaccine and two of these four patients remained in SD throughout the study. Six patients had early termination of the study, i.e. before 2nd CT scan. Ten patients completed all ten planned vaccinations. One patient proceeded in off study treatment and received additional monthly vaccinations due to continuous SD. The clinical benefit response rate was $\sim 23\%$ (4/17).



2.3.2. CRC Singapore

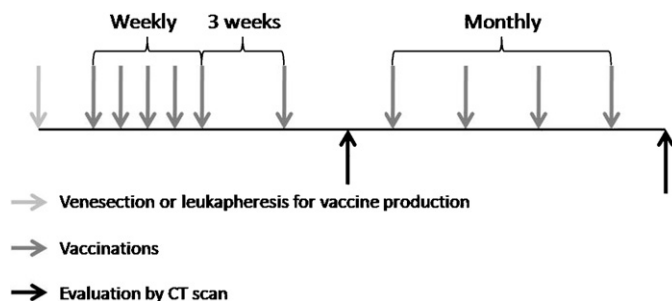
Twenty patient with progressing CRC stage III and IV showing a WHO performance status ≤ 2 were enrolled and 12 patients received a full vaccine program of 10 biweekly vaccines. Data from this study are accepted for publication (Toh C.H. et al.). One patient experienced a partial response (PR) and seven patients displayed SD and the clinical benefit response rate was 40% (8/20). Study design as shown for CRC Denmark.

2.3.3. NSCLC Denmark

Twenty-eight patients with advanced NSCLC and a WHO performance status ≤ 2 were enrolled in this study and 22 patients initiated treatment. Sixteen patients were evaluable and seven patients received all 10 vaccines. Four patients proceed in off study treatment and two patients are still active after more than 2 years in the protocol and off study. Seven patients displayed SD and the clinical response rate was $\sim 32\%$ (7/22).

In all three clinical trials patients were evaluated according to RECIST criteria.

2.3.4. Study design



2.4. Statistical analysis

Comparison of samples to establish the statistical significance of difference was determined by unpaired two-tailed Student's *t*-test. Results were considered to be statistical significant when $p \leq 0.05$.

3. Results

3.1. CRC phase II (Denmark)

All DC vaccine batches generated from CRC patients included in the Danish clinical trial were as described in Material and Methods generated using the first generation of the DDB DC protocol and maturation stimulus was provided by TNF α . A total of 36 batches were generated for 17 patients.

The DC batches generated from these patients displayed a semi-matured phenotype and we observed batch-to-batch variations regarding number of cells expressing each surface marker included in the FACS analysis. DC gated by FCS and SSC expressed high levels of CD86 (84% positive cells), and HLA-DR (96%) concomitantly with a medium level of CD1a (52%) and CD83 (36%). However, only a low fraction of the cells expressed CCR7 (14%). Some patient DC had high levels of CD14 positive cells (>50%) on day 7 after maturation stimulus which correlated with low CD83 expression.

3.2. CRC phase II (Singapore)

DC generated from the CRC patients included in the Singapore trial was all matured with the cytokine cocktail: IL-1 β , IL-6, TNF α and PGE $_2$. A total of 38 DC batches were generated for 20 patients. DC gated by FCS and SSC showed a significantly higher expression of both CD83 (74%), CD86 (97%) and CCR7 (59%) concomitantly with high expression of and HLA-DR (99%) compared to DC batches used in the Danish CRC trial (Fig. 1). CD14 was expressed to a similar level in these DC batches (22%) compared to the batches in the Danish CRC trial. Thus, we observed an increase in the expression of the maturation surface markers in DC generated from the Singapore patient cohort compared to DC batches generated for the Danish CRC trial (Fig. 1). We again observed batch-to-batch variations in the vaccine generation. Not surprisingly the cytokine cocktail yields DC batches with a more mature phenotype as demonstrated previously for health donors [20] thus emphasizing that this is true also when generating monocyte-derived DC from CRC patients. Within the two CRC patient cohorts we compared DC batches generated from patients who displayed clinical responses with DC batches generated from patients without a clinical response. We did not

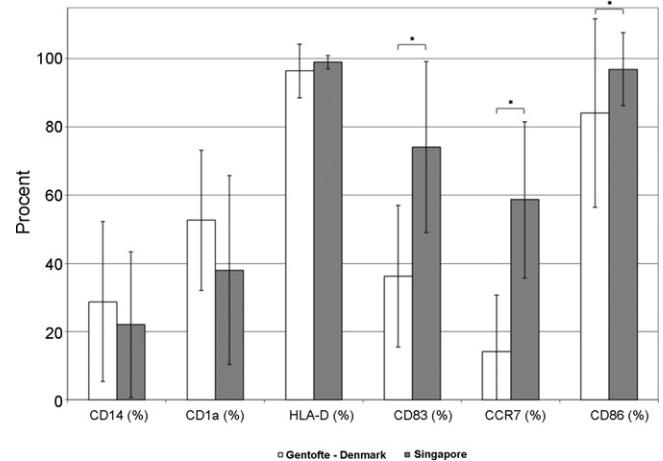


Fig. 1. CRC Denmark and Singapore. Comparison of expression of surface markers on Mo-DC generated from CRC patients. DC from patients in Denmark are generated using the first generation of the DDB DC method and 36 DC batches generated from 17 patients have been included. The DC from patients in Singapore are generated using the second generation of the method. A total of 38 DC batches generated from 20 patients have been included. Data are presented as mean \pm S.D. * $P < 0.05$ for two tailed unpaired Student's *t*-test.

find differences in the surface marker expression between these DC batches in either patient cohort (Denmark and Singapore) which indicates that monocytes from each group were equally susceptible to the cytokine stimulation (Fig. 2).

3.3. Comparison of DC profiles from batches generated before and during treatment

We looked at the each DC batch generated from the clinical responders separately, and found that the maturation status of the DC generated from the CRC, SG patient achieving a PR improved from the first to the second batch. The first batch was generated prior to treatment and the second after the 4th vaccine. We observed a significant increase in the expression of CD83 and CCR7. Even though this is only based on the two time points available, it indicates that monocytes harvested from this patient during the treatment were more susceptible to the cytokine exposure and thereby attained a more optimal mature profile compared to monocytes isolated prior to treatment. The general picture for the patients achieving SD was random or even decreasing expression of most markers. Data from the DC batches from the patient with PR

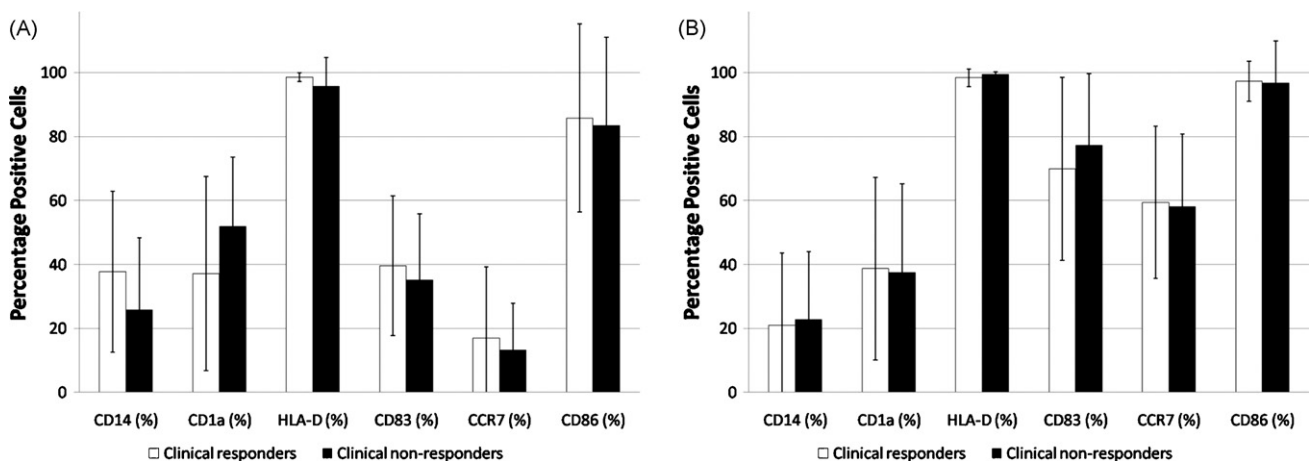


Fig. 2. Comparison of DC batches generated from clinical responders and non-responders in the two trials with CRC patients. A: CRC Denmark, average of 36 DC batches SD: $n = 9$, PD $n = 27$, B: CRC Singapore, average of 38 DC batches SD/PR: $n = 16$, PD: $n = 22$. Data are presented as mean \pm S.D.

Table 1
Expression of surface markers from DC batches over time.

I.d.	CD14 (%)	CD1a (%)	HLA-D (%)	CD83 (%)	CCR7 (%)	CD86 (%)
PR, 1	2.15	5.93	94.62	30.86	26.90	99.05
PR, 2	4.06	19.25	99.86	96.80	83.42	99.75
SD, 1	2.14	63.97	98.50	90.52	62.10	95.71
SD, 2	28.80	48.33	96.57	51.36	46.12	93.07
SD, 1	36.21	2.98	99.94	66.61	62.03	99.78
SD, 2	26.17	1.73	89.69	28.08	34.24	75.23
SD, 3	62.94	3.78	99.69	37.46	38.02	99.26

and batches from two representative patients with SD are shown Table 1.

3.4. NSCLC phase II

A total of 35 DC batches were included generated from 22 patients. DC batches generated with the cytokine cocktail mentioned above, from the patients with NSCLC displayed high expression of CD86 (95%) and HLA-D (94%) and intermediate expression of CD83 (63%), CD1a (37%) and CCR7 (45%). Average expression of CD14 was 23%. Also in these DC batches we observed a large batch-to-batch variation between all included batches both inter- and intra-patient (Fig. 3).

3.5. Comparison of DC batches generated from two different types of cancer

When comparing DC batches generated from CRC Singapore patient cohort and from NSCLC patient cohort, it is a comparison of the second generation DDB DC protocol used in two different types of cancer (Fig. 3). The average of percentages of positive cells expressing CD14, CD1a, CD86 are comparable, however, the trend is that there is a decrease in percent positive cells for some markers in DC generated from NSCLC patients compared to the CRC patients. For CD83 and CCR7 the decrease from CRC DC to NSCLC DC is significant. This observed trend in decreased maturation status of DC from NSCLC patients indicates that monocytes from some NSCLC patients are less susceptible to the cytokine-induced maturation compared to monocytes from CRC patients.

Within DC batches from NSCLC patients we observed a trend towards more optimal mature DC expression pattern from patients with SD compared with DC profiles from patients with progressive disease suggesting that the degree of susceptibility to

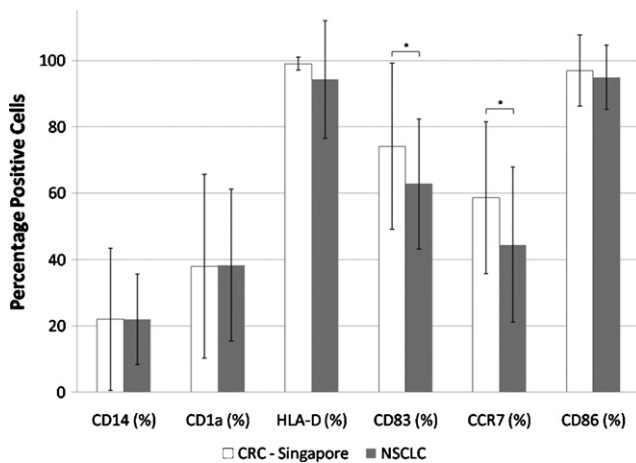


Fig. 3. Comparison of DC batches generated using the second generation method from monocytes from CRC patients (38 DC batches from 20 patients) and NSCLC patients (35 DC batches from 22 patients). Data are presented as mean ± S.D. *P < 0.05 for two tailed unpaired Student's *t*-test.

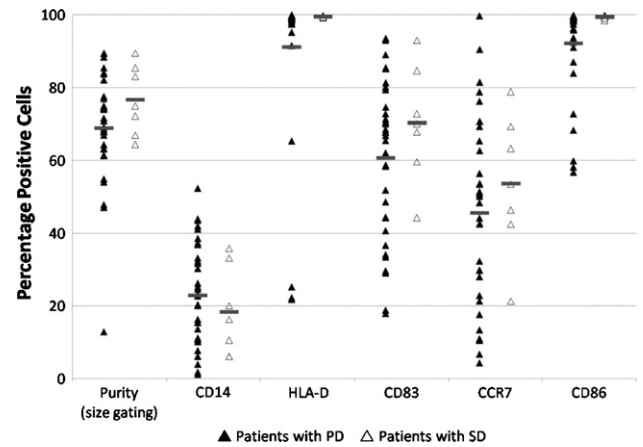


Fig. 4. Comparison of DC batches' FACS profiles between clinical responders (SD) and non-responders (PD) of the NSCLC patient cohort. Each triangle represents a DC batch. Black bars indicate the average expression of each marker in each group (SD, PD). SD, *n* = 7 and PD, *n* = 28.

cytokine stimulation depends on the clinical status of the patients (Fig. 4).

3.6. Comparison of DC batches from cancer patients and healthy donors

Finally we have made a phenotypic comparison between DC batches from cancer patients (Singapore CRC and NSCLC) and healthy donors, all generated using the second generation DC protocol (Fig. 5). This comparison shows that monocytes from healthy donors reach a significantly higher maturation stage in terms of surface marker expression compared to DC generated from either cohort of cancer patients. The expression of CD14, CD83 and CCR7 is significantly improved and we observed less batch-to-batch variation compared to samples from cancer patients.

4. Discussion

We have generated autologous monocyte-derived DC (Mo-DC) based vaccines for immune therapy from three different cancer patient cohorts; two cohorts of patients with CRC and one cohort

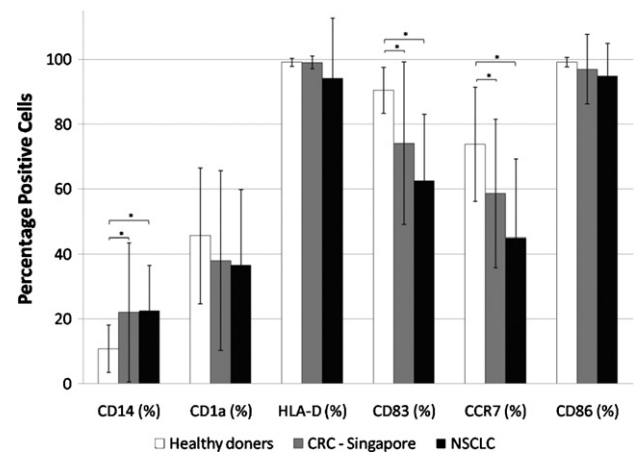


Fig. 5. Comparison of surface marker profiles of DC generated from cancer patients and healthy donors. We found a significant increase in the expression of CD83 and CCR7 and a significant decrease of CD14 in healthy donors Mo-DC compared to cancer patient Mo-DC. Data are presented as mean ± S.D. *P < 0.05 for two tailed unpaired Student's *t*-test. Healthy donors, *n* = 13, CRC Singapore, *n* = 38, NSCLC, *n* = 35.

of patients with NSCLC. In the present paper we retrospectively compared the maturation profile based on surface marker expression between the three patient cohorts and between cancer patient cohorts and a cohort of healthy donors.

Vaccines were generated under cGMP conditions and phenotypic profiles of Mo-DC were analyzed by flow cytometry and the obtained data were used to set guideline values for our quality control of GMP produced DC vaccine. Each vaccine batch was analyzed for the expression of the surface molecules CD14, CD1a, CD83, CD86, MHC class II and CCR7, and we considered the optimal expression pattern to be CD14^{low}, CD1a, CD83^{high}, CD86^{high}, MHC class II^{high} and CCR7^{high}.

4.1. Functionality of DC

Functionality of the DC generated for the clinical trials was assessed by immune monitoring of the vaccinated patients. Plasma samples from patients included in the CRC Denmark trial were analyzed and the results showed that patients with clinical benefit (SD) had a significant increase in the level of GM-CSF, TNF α , INF γ , IL-2 and IL-5, suggesting vaccine-induced Th1 responses [21]. DTH tests were conducted after the 5th and 10th vaccine on patients included in the CRC Singapore trial and positive reactions were observed in 8 of 15 patients after the 5th vaccine and in 9 of 12 patients after the 10th vaccine. In addition, the frequency of regulatory T cells defined as CD4⁺CD25^{hi}CD127^{-lo}Foxp3⁺ was monitored and a decline in these cells was observed during the treatment. However, DTH responses and the decline in regulatory T cells did not correspond to clinical outcome (Toh C.H. et al., Manuscript in accepted for publication). PBMC samples were collected from patients enrolled in the NSCLC trial to conduct cellular immune monitoring using vaccine-specific release of IFN γ as a read out analyzed on single cell level by EliSpot. We found vaccine-specific IFN γ release in 5/7 patients displaying SD during treatment and in 1/13 patients with PD. Thus we detected a statistically significant correlation between clinical outcome and immune responses in these patients ($p=0.0072$, two-tailed Fisher's Exact test) (Kvistborg P. et al., Manuscript in preparation).

4.2. Quality of our Mo-DC

Our findings demonstrate that there are differences in the quality of the Mo-DC dependent on the type of cancer, and that monocytes from cancer patients are less susceptible to cytokine stimulation compared to monocytes from healthy donors. Furthermore we observed that the maturation status of the DC correlates positively with the clinical status of the patients. When the cancer is diagnosed at the advanced stage, the median survival for CRC patients is 20 months following therapy with conventional cytotoxic chemotherapy [22] whereas it is only 8–10 months for NSCLC patients [23]. This indicates that NSCLC is a more aggressive disease compared to CRC suggesting that NSCLC might be a more immune suppressive disease than CRC. Accordingly, DC batches generated from Singapore CRC patients express significantly higher levels of the maturation marker CD83 and the chemokine receptor CCR7 compared to DC batches from NSCLC patients. In addition, the trend was that Mo-DC generated from NSCLC patients with an overall better clinical status (SD compared to PD) displayed a more mature phenotype indicating that this subpopulation of patients were generally less immune suppressed.

4.3. Effects of different maturation stimuli

When using the first generation of the DDB DC protocol we observed that CCR7 expression was very low with an average of 14% positive cells per DC batch. The ligands for the CCR7 receptor,

the chemokine CCL19 and CCL21 are primarily produced in T cell rich paracortical areas of lymph nodes [24,25]. Hence the expression of CCR7 on Mo-DC for vaccine purpose is a prerequisite for the induction of an effective immune response [26]. The cytokine cocktail (IL-1 β , IL-6, TNF α and PGE₂) is known to have a positive effect on the CCR7 expression [27] and therefore it was implemented in the second generation of the DC protocol. We observed a significant increase in the CCR7 expression from the DC batches generated from the Danish CRC trial compared to the DC batches generated in the Singapore CRC trial, emphasizing the effect of in particular PGE₂ on the CCR7 expression.

4.4. DC in cancer patients

It is established that DC isolated from cancer patients are dysfunctional. Three studies including breast cancer patients have compared functionality and phenotype with stage of the cancer, and report that functionality and expression of maturation markers decrease with advanced stages of cancer [8,10–12]. The reason for the observed defective function in these DC is most likely due to abnormal differentiation of myeloid cells [28]. This abnormal differentiation has at least three main consequences, namely decreased production of mature functionally competent DC. The accumulation of immature DC that have characteristics of DC but cannot upregulate MHC class II and costimulatory molecules or produce appropriate cytokines, and increased production of immature precursor cells [28]. DC isolated from peripheral blood and lymph nodes were compared and the results showed that defective DC are not restricted to local tumor draining lymph nodes but rather is a systemic occurrence [10], and this suggests that it is mediated by soluble factors produced by tumor cells. This was confirmed by the observation that *in vitro* differentiation of precursor cells into DC could be inhibited by addition of conditioned medium from *in vitro* tumor cell cultures [29,30]. Apoptotic tumor cells are the source of tumor antigens to be presented to T cells by DC to induce anti-tumor immunity. However, DC might become dysfunctional subsequent to interacting with apoptotic tumor cells depending on how tumor cells became apoptotic [31].

4.5. Mo-DC generated from cancer patients

In line with the results of these studies, it has been demonstrated that monocytes from cancer patients are influenced by tumor factors rendering them less susceptible to cytokine induced differentiation and maturation. Gabrilovich et al. showed that Mo-DC from breast cancer patients displayed comparable phenotype to Mo-DC from healthy donors, however, the Mo-DC from cancer patients showed decreased capability to induce cytotoxic activity in T cells [8]. Another study showed that uptake of apoptotic bodies was significantly lower in Mo-DC from cancer patients compared to Mo-DC from healthy donor and CD80 and MHC class I expression was decreased in cancer patient Mo-DC compared to HD Mo-DC. In addition, Mo-DC from healthy donors showed significant increase in allogeneic T cell stimulation [32]. Pedersen et al. showed that Mo-DC from breast cancer patients displayed comparable phenotype to HD Mo-DC, however, CD54 and CD40 expression was significantly elevated in the cancer patient Mo-DC. Mo-DC from cancer patients induced lower allogeneic T cell stimulation and the authors concluded that cancer patient Mo-DC were more differentiated but less sensitive to maturation-inducing agents than Mo-DC obtained from healthy donors [33]. Thus, the conclusion on these and similar studies is an observed trend indicating that Mo-DC from breast cancer patients yield less optimal phenotype and functionality compared to healthy donor Mo-DC [34]. The variation on how this is defined may be due to variations in DC generation. This correlate with what we have observed for Mo-DC generated

from CRC and NSCLC patients. Another study has demonstrated that Mo-DC from paediatric cancer patients and healthy donor Mo-DC were comparable both regarding phenotype and functionality [35]. These results correlates well with the assumption that paediatric cancer patients in general is less immune suppressed due to their young age [35].

In summary we have made a comparison of Mo-DC phenotypes generated from two types of cancer, i.e. CRC and NSCLC and to our knowledge we are the first to report on a comparison of DC phenotypes from these two cancer types. We have shown that the quality of Mo-DC depends on type of cancer and the clinical status of the cancer patient. However, even though the quality of Mo-DC from cancer patients is decreased compared to Mo-DC from healthy donors, they are phenotypically and functionally superior to *in vivo* DC in cancer patients and thus Mo-DC are relevant as adjuvant in cell-based cancer vaccine therapy. However, concurrent with results showing that stage of cancer directly correlates to the degree of immune suppressive status of the patients it will be advantageous to select cancer patients for immune therapy on the basis of their immune status by the use of DC phenotype scoring and tumor biomarkers to increase the chance for clinical benefit. More promising clinical results are expected when DC-based immune therapy moves into groups of cancer patients in the earlier stages of cancer.

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